DIFFERENT CLASSES OF INITIATION FACTORS F3 AND THEIR DISSOCIATION ACTIVITY

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1. Introduction

The initiation of protein synthesis in *E. coli* requires several protein factors which are loosely associated with ribosomes. Three initiation factors: F1(A), F2(C) and F3(B) have already been described [1–3].

The initiation step in which F3 is directly involved is not clear as yet. Requirement for F3 in translation was first reported for systems involving naturally occurring, as opposed to synthetic, messengers [4–6]. Hence it has been postulated that F3 represents some kind of specific "recognition" factor permitting ribosomes to start translating natural messengers at defined sequences. Although direct F3 binding to purified messenger RNA has been reported [4, 6, 7] it cannot as yet be assessed whether this type of interaction, outside ribosomes, is specific and related with the initiation factor activity proper.

Ability to dissociate 70 S ribosomes into their subunits (DF activity) [8–13] has also been ascribed to F3 since homogeneous preparations of this factor were shown to exhibit the property to stimulate both messenger directed fMet-tRNA binding and ribosome dissociation [12, 13]. In short, F3 would have a dual function: 1) to dissociate ribosomes into their subunits and 2) to stimulate the formation of the initiation complex on dissociated 30 S subunits. In contrast, it has been reported that the dissociating activity could be separated from crude initiation factors on DEAE-cellulose and urea column, and might be associated with F1 [14].

The present communication confirms previous reports according to which F3 can be fractionated into subclasses differing in their ability to recognize various natural messengers, thus giving support to the "messenger selection" function of this factor [15].

It none the less raises some new questions regarding the nature, properties, as well as the role of F3 factors since it is observed that: (1) F3 fractions specific for RNA phage messengers also respond to synthetic messengers (such as poly U, G) or synthetic triplets (such as GpUpG). Similar observations have independently been made by Dubnoff and Maitra [13]; (2) certain F3 subfractions only respond to poly A, U, G or ApUpG exclusive of other messengers; and (3) not all F3 fractions (especially those responding to T4 mRNA or poly A, U, G) exhibit DF activity. The possibility that "initiation triplets recognition" factors distinct from "sequence selection" factors do exist is discussed.

2. Material and methods

Preparative procedures for obtaining poly A, U, G, poly U, G, ApUpG, GpUpG, T4 mRNA, as well as

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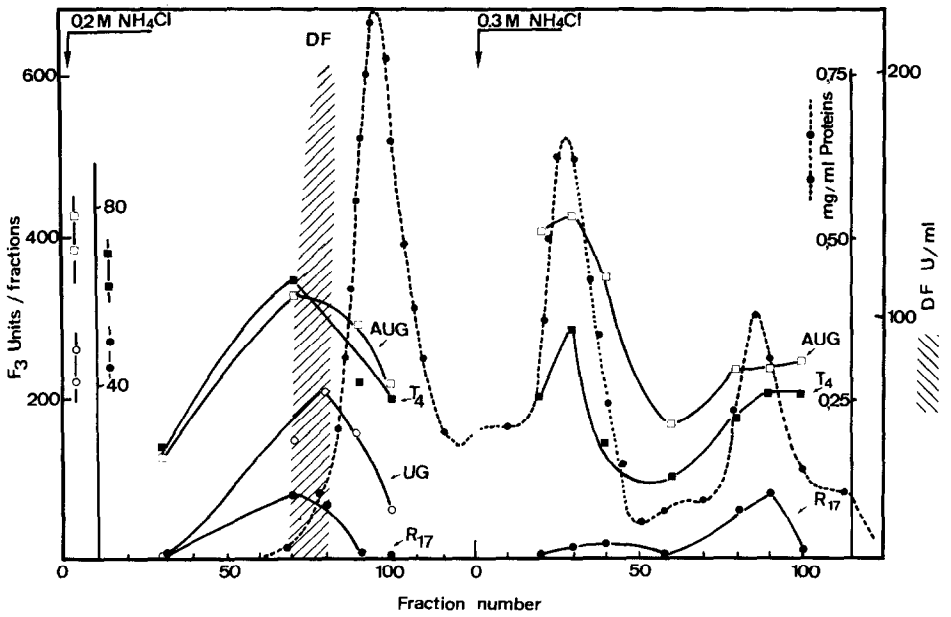

Fig. 1. Elution pattern from DEAE-cellulose column of initiation factor F3 (F3b and F3c) specific for different messengers. The incubation mixture for F3 assay (100 μl) contains: 50 mM Tris, pH 7.5; 80 mM NH_4Cl; 7 mM Mg acetate; 1 mM GTP; 2 A_{260} units ribosomes; 2.7 μg factor F1; 4.5 μg factor F2; 1 A_{260} unit specific activity 620 cpm/mole ^{3}H-fMet-tRNA; 0.3 A_{260} unit synthetic mRNA; natural mRNA, 2 A_{260} units; plus 30 μl fractions of F3 (in 20 mM K_3PO_4 buffer). The final PO_4 concentration was always kept at 6 mM. Filtration on nitrocellulose membranes (Millipore) was performed as previously described [17]. 1 unit of F3 correspond to 1 pmole fMet-tRNA bound after the amount bound by F1 + F2 has been substracted. Saturation for F1 + F2 is obtained with 1.3 μg of F1 and 2.3 μg of F2; increasing F1 up to 4 μg and F2 up to 7 μg does not change the pattern. The amounts of fMet-tRNA bound by F1 + F2 were as follows with different mRNAs: A, U, G, 2.9; U, G, 0.41; T4, 0.2; R17, 0.156. The cross-hatched part corresponds to DF (see table 2 for DF test).

crude initiation factors [16], or purified F1 and F2 and fMet-$tRNA_f$ have been previously reported [17].

R17 and Qβ mRNA were, respectively, gifts from Dr. Cornuet (CNRA Versailles, France) and from Dr. Kolakofsky (Inst. Mol. Biol., Zurich, Switzerland).

2.1. *Preparation of F3 subfractions*

Three main F3 subfractions (which will be termed F3a, F3b and F3c) have been prepared and their biological properties compared in using appropriate messenger RNAs. These subfractions originated from 700 ml of a crude initiation factor mixture (IF) from *E. coli* MR 600 containing 2.8 mg protein/ml.

2.2. *Crude F3a*

Crude IF was submitted to differential $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 0–30% saturation (5% proteins recovered) $(NH_4)_2SO_4$-0) and the one obtained between 30–45% saturation $(NH_4)_2SO_4$-I (30% recovery) were pooled and dialyzed against buffer A (20 mM K_3PO_4, pH 7.5; 5% glycerol; 7 mM β-mercaptoethanol). The dialysate was adsorbed on a DEAE Sephadex A-50 column (3.5 × 45 cm). After washing with buffer A, proteins were eluted with a linear salt gradient: 2 × 500 ml buffer A, containing 0.02 M and 0.2 M NH_4Cl, respectively (6 ml fractions were collected). F1 was recovered between tubes 120 and 190.

F3 was then eluted: first by washing the column with 1200 ml buffer A containing 0.2 M NH_4Cl (tubes 280–400) and further by one liter of gradient made with buffer A containing 0.2–0.4 M NH_4Cl (tubes 420–440) ("crude F3a").

F2 was eluted later with 0.4 M NH_4Cl in buffer A (tubes 500–720).

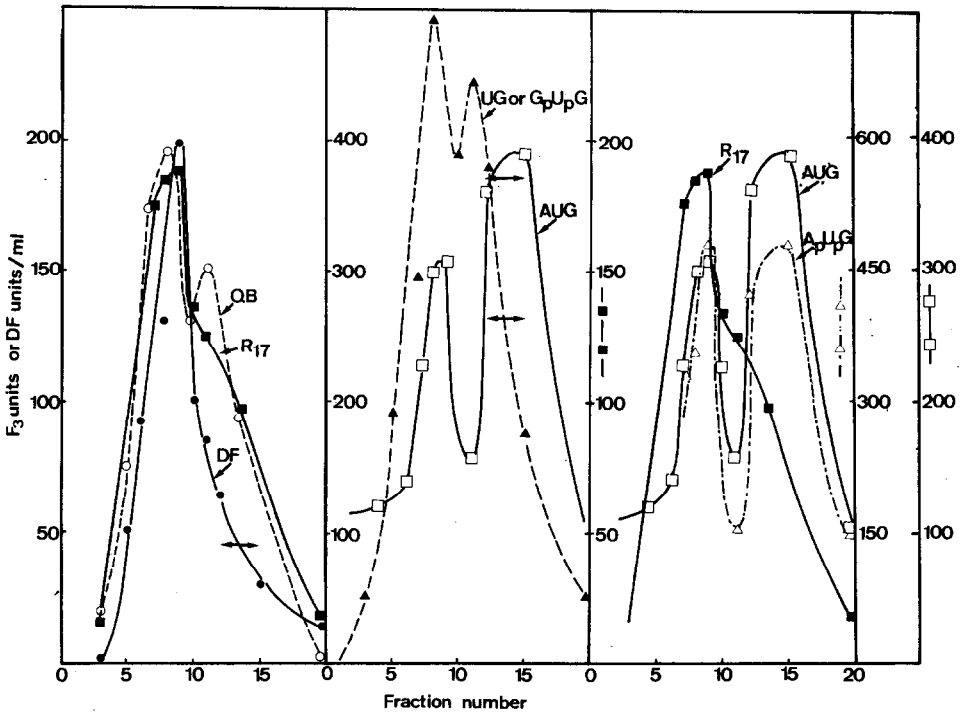

Fig. 2. Comparison of DF activity and F3 activity with different mRNAs with fractions eluted from phosphocellulose column 2. Same test conditions as in fig. 1, except for the fMet-tRNA specific activity, which was 6650 cpm/mole. Each point is the average of several tests using different amounts of F3 (2–25 μl) to make sure that the linearity as a function of protein was repeated.

2.3. *Crude F3b and F3c*

The fraction precipitating from the IF mixture between 45–75% $(NH_4)_2SO_4$ saturation (38% proteins recovered) $(NH_4)_2SO_4$-II) was dialyzed against buffer A plus 2 mM Mg acetate before being adsorbed on a DEAE cellulose column Whatman DE-52 (h = 45 cm, diameter: 3.5 cm). After washing with 700 ml buffer A plus 2 mM Mg acetate, "crude F3b and F3c" were separated by stepwise elution using 1 l buffer A containing 0.2 M and 1 l buffer A containing 0.3 M NH_4Cl. 7 ml fractions were collected (fig. 1).

2.4. *Purified F3b and F3c*

Crude F3b was purified by 2 successive chromatographies on phosphocellulose (PC) columns: fractions 30–110 of the 0.2 M NH_4Cl eluate were pooled (93 mg protein), dialyzed twice against 4 l of buffer A and adsorbed on a first PC column (Mannex: 20 × 1.5 cm). Washing with 250 ml 0.05 M K_3PO_4 buffer, pH 7.5, removed 87% of proteins devoid of F3 activity. F3 was eluted by establishing a phosphate gradient (2 × 150 ml) containing between 0.05 and 0.8 M K_3PO_4, pH 7.5; glycerol, 5%; and β-mercaptoethanol, 7 mM; 2 ml fractions were collected (fig. 2).

The DF activity is eluted from tubes 45–90 while F3 activity with poly A, U, G or phage mRNA is eluted from tubes 60–110 (not shown).

Tubes 61–110, containing most of the F3 activity and roughly half the DF activity, are pooled (207 mg of proteins) and dialyzed 18 hr against buffer A; they are then placed upon a second phosphocellulose column (3 × 1 cm) equilibrated with the same buffer. The proteins are eluted by a linear gradient of 15 ml 0.2 M K_3PO_4, pH 7.5 and 15 ml of 0.75 M K_3PO_4, pH 7.5, containing 5% glycerol and 7 mM β-mercaptoethanol; 1 ml fractions were collected, containing an average of 60 μg/ml protein.

"Crude F3c" was dialyzed twice against 4 l of

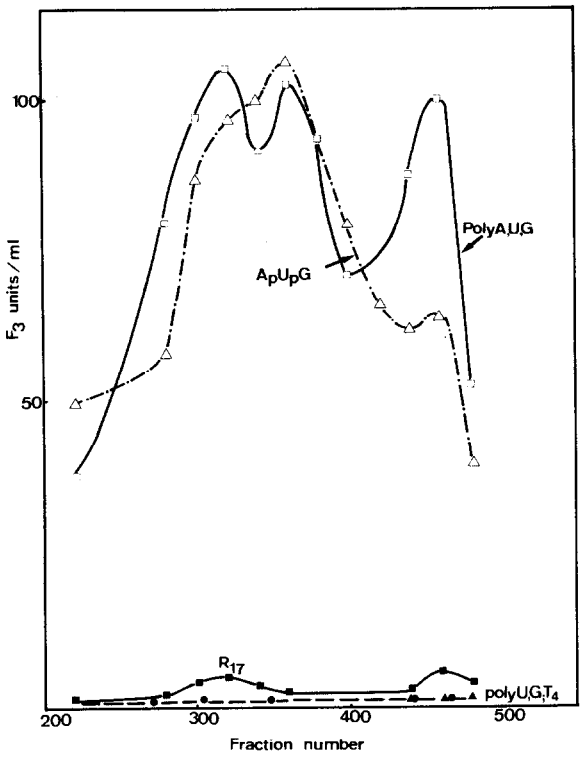

Fig. 3. Specificity of F3a towards different mRNAs. The amounts of fMet-tRNA bound by F1 + F2 were as follows with different mRNAs: poly A, U, G, 4.77; poly U, G, 1.148; ApUpG, 2.6; T4, 0.654; R17, 0.965. Binding conditions as in fig. 1 with 30 μl fractions of F3 (in 0.02 M buffer) containing between 1.5–3.4 μg protein.

buffer A for 24 hr and purified by PC column fractionation as described for F3b (same conditions as for the first PC column). Fractions of 2 ml each were collected. Fractions 50–130 were pooled, and concentrated by Diaflo (PM10 membrane), yielding "purified F3c".

2.5. *Determination of ribosome dissociating factor activity*

As in legend of table II.

3. Results

3.1. *Comparative initiator tRNA binding activities of crude F3 subfractions (F3a, b, c)*

F3 subfractions, F3a, F3b, F3c, partially purified on DEAE cellulose or Sephadex as above, have been compared for their abilities to catalyze fMet-tRNA binding to ribosomes in the presence of various phage or synthetic messengers.

As shown in fig. 3, F3a exhibits a remarkable specificity. It highly stimulates initiator tRNA binding when poly A, U, G or ApUpG triplet are used as templates (F1 and F2 being present in saturating amounts); whereas with other mRNAs, including synthetic poly U, G, no stimulation is observed. This poly A, U, G or ApUpG-dependent stimulation is the same whether 70 S ribosomes or 30 S + 50 S subunits are used, indicating that it cannot be accounted for solely by ribosomal dissociating activity.

Partially purified F3b, as derived from the $(NH_4)_2SO_4$-II precipitate and prepared from the DEAE cellulose fractions eluted at 0.2 M NH_4Cl (fig. 1), shows a different activity spectrum with respect to the kind of messenger involved: initiator tRNA binding activity is observed with poly A, U, G, poly U, G, T_4, Qβ, and R17 RNAs.

Finally, F3c corresponding to fractions eluted from the DEAE cellulose column at 0.3 M NH_4Cl is virtually inactive with R17 mRNA and poly U, G but responds quite appreciably to T_4 mRNA and to poly A, U, G.

3.2. *Properties of "purified" F3b and F3c*

Crude F3b and F3c fractions have been further purified by phosphocellulose chromatography (see Material and methods).

"Purified F3b" still appears heterogeneous with respect to its messenger dependency (fig. 2): when natural mRNA, poly U, G or GpUpG are used as templates 2 main activity peaks are observed and the activity with these different messengers seems to be associated with the same chromatographic fractions. In a poly A, U, G or ApUpG dependent assay F3b activity also shows 2 peaks but these are in two clearly separated chromatographic fractions which behave differently in R17 mRNA or poly U, G-dependent test.

The "purified F3c" (table 1) exhibits a higher activity with poly A, U, G and T_4 mRNA than with other mRNAs, confirming results obtained with the same fraction in a crude state.

It must be noted that none of the F3 subfractions have the property of stimulating Phe-tRNA binding

Table 1
Comparison of F3 activities with different templates.

F3 fractions	$Poly_{AUG}$	T_4	R17	T_4/R17
$(NH_4)_2SO_4$-II (19 μg)	4.25	1.1	1.39	0.8
F3a (1.92) tube no. 340	2.76	0	0.062	0
F3b (1.32 μg) tube no. 9	1.54	0.86	1.17	0.72
Pool 12–15 (1 μg)	3.12	1.58	1.14	1.4
F3c (9 μg)	1.37	0.247	0.03	8

Conditions as in fig. 2. Values found with F1 and F2, were as follows (in pmole): T4, 1.65; R17, 0.95; poly A, U, G, 6.99.

in the presence of poly U (whether elongation factor T and/or factors F1 + F2 are added or not to the assay mixture).

3.3. *DF activities of F3 subfractions*

The abilities of the various F3 preparations to promote the dissociation of 70 S ribosomes into their subunits, as probed by the sucrose gradient sedimentation technique, have been examined and the following was observed: (1) A high DF activity could be shown in the 0.2 M NH_4Cl eluate (crude F3b) and this activity was distributed in a peak much sharper than that corresponding to the fMet-tRNA binding (F3) activity (fig. 1). (2) Phosphocellulose purified F3b fraction clearly exhibited DF activity. This activity roughly followed the fMet-tRNA binding activity tested with R17 mRNA, poly U, G or GpUGp, although coincidence was not absolute. The tube with the highest DF activity (no. 9) (fig. 2) had a specific activity about 100 times greater than that of the starting $(NH_4)_2SO_4$-II fraction (table 2). In the poly A, U, G or ApUpG-dependent fractions the one materialized by the second activity peak had very little DF activity. (3) No DF activity could be detected among the various fractions of the 0.3 M NH_4Cl eluate containing F3c activity.

Table 2
Purification of ribosome dissociating activity (DF).

Fractions	Total protein (mg)	Total units	Units/mg
Crude initiator factor	1960	70,000	36
$(NH_4)_2SO_4$-0	108	0	–
$(NH_4)_2SO_4$-I DEAE cellulose F3a	657	16,000	25
$(NH_4)_2SO_4$-II	741	19,000	27
DEAE cellulose 0.2 M NH_4Cl pool 30–110	83	10,400	125
Phosphocellulose-I pool 61–110	3.3	2,600	804
Phosphocellulose-II F3b fraction 9	0.066	200	3030
pool 12–15	0.21	280	1346

1 unit of DF is the amount necessary to dissociate 1.3 A_{260} units of 70 S ribosomes so that the 50 S/70 S ratio is 1/1. Reaction mixtures were prepared at 0° and contained the following components in a total volume of 0.2 ml: 25 mM Tris-Cl buffer, pH 7.8; 50 mM KCl; 6.75 mM Mg-acetate; 2 mM β-mercaptoethanol; 2.3 mM GTP; 1.3 A_{260} units ribosomes; 20 mM NH_4Cl; 3 mM K_3PO_4; and variable amounts of protein to be tested for DF activity. The tubes were incubated at 25° for 20 min and were then cooled in an ice bath. For analysis of the ribosomes species, 0.1 ml of the chilled mixture was layered on a 3.8 ml 10–30% linear sucrose gradient (prepared from Mann ultrapure dialyzed sterile 50% solution) containing 10 mM Tris-HCl, pH 7.8; 50 mM KCl, and 5 mM Mg-acetate, equilibrated for 24 hr at 4°. The gradients were centrifuged for 90 min at 50,000 rpm in a Spinco SW 56 rotor. The A_{260} of the gradients was determined with the expanded scale of a Cary 14 by pumping the contents of the tube through a Gilford 2 mm flow cell. The ribosome preparation contained almost exclusively 70 S particles, but the presence of 5–10% of 30 S or 50 S particles would not have been detected.

3.4. *Properties of DF and F3*

3.4.1. Effect of GTP on DF activity

GTP was initially added to the incubation mixtures because it was found to stimulate DF activity of the samples. This stimulating effect already observed by Algranati [10] and Dubnoff and Maitra [13],

could, however, be accounted for by the chelating action of GTP on Mg^{2+} ions.

Under the test conditions used, 70 S ribosomes were completely dissociated when incubated at 25° for 20 min in the absence of added Mg^{2+}; at 2 mM Mg^{2+} no significant dissociation was detected in the absence of added DF. The extent of DF mediated dissociation (using tube fraction 9) was the same in an incubation mixture containing no GTP but 3 mM Mg^{2+}, and 5.3 mM Mg^{2+} plus 2.3 mM GTP.

3.4.2. Linearity of F3 activity with protein concentration

Up to 1–1.5 units (above which an inhibition occurs) F3 activity increases linearly with the protein concentration; this is true with fractions F3a and F3b, fraction F3c being inactive at low protein concentration.

Addition of purified DF (tube 9) to F3c (which is devoid of DF activity) does not result in a stimulation of the fMet-tRNA binding capacity, as compared to what is observed when each component is tested separately.

4. Discussion

The purpose of the present study was to purify the protein fraction F3 which is usually believed to represent one single class of the initiation factors involved in RNA translation. Two kinds of biological tests were used, one based on the capacity of F3 to complement F1 + F2 in messenger directed binding of fMet-tRNA, the other on the previously described property of F3 to promote dissociation of 70 S ribosomes (DF activity).

Our results point towards the difficulty of defining F3 by a specific and unequivocal criterion but do suggest that what is usually designated as F3 consist either of different types of proteins or of aggregates between F3 and other proteins or between F3s.

4.1. *F3 and DF activity*

Insofar as the dissociating activity of F3 is concerned, a protein fraction with DF activity has been purified about 100-fold by successive chromatography procedures. Despite the fact that some purified F3 fractions (F3b, tubes 7–10, fig. 2) contain a strong DF activity, it is clear from our results that coincidence between DF and F3 activities is far from being systematic. In particular, DF activity of crude F3b (see fig. 1) is located, after elution, in a very symmetrical peak whereas F3 activity, as measured by the initiator tRNA binding assay is much more polydispersed. Phosphocellulose purification of F3b reveals a fairly good coincidence between DF and F3 activities when the latter is assayed with R17 mRNA or with poly U, G; in contrast, the poly A, U, G or ApUpG responding subfraction shows very little DF activity. Uncoupling between DF and translation–initiation activity is confirmed by examining crude, or phosphocellulose-purified F3c; this subfraction, although clearly active in stimulating poly A, U, G or T4 mRNA-directed fMet-tRNA binding, cannot promote any 70 S dissociation. Purification of F3 fractions without DF activity has independently been achieved by Bosch et al. [18].

A possible explanation for the fact that some F3 fractions exhibit a dissociating activity while others do not, might be found in the assumption that ribosomes do not have a stable, rigid structure, as already suggested [19] but can undergo conformational changes, particularly when interacting with initiator factors. The fact that initiation protein, when mixed with a 30 S ribosomal subunit hinders its ability to bind streptomycin would be in favour of a factor-mediated conformational change [20]. The possibility thus exists that binding of F3 to the 30 S moiety of a 70 S dimer can change the ribosomal conformation in such a way that, under test conditions, a dissociation can be observed. With certain classes of F3 subfractions, such as the one specific for poly A, U, G, ApUpG, or T4 mRNA, factor binding would alter ribosome structure without causing its dissociation. This would perhaps imply that "non-dissociating factors" operate in conjunction with those endowed with dissociating activity. Alternatively, the phenomenon of F3-directed ribosome dissociation, which has mostly been observed under special conditions (low Mg^{2+} and strong hydrodynamic forces to unravel the dissociated subunits) might represent some kind of artefact. The relevant effect of F3 factors could be restricted to a conformational alteration of ribosome, prior to formation of an initiation complex. If such were the case, the existence of F3 factor, devoid of true dissociating activity, would raise no theoretical difficulty.

4.2. *Messenger specificity of F3*

The results of this investigation show that F3-dependent stimulation of fMet-tRNA binding (F1 and F2 being present in saturating amounts) is not only observed with natural messengers such as R17, Qβ, or T4 mRNA, but also with synthetic polymers (poly A, U, G, poly U, G) and even with specific triplets, ApUpG or GpUpG. (F3 dependence on synthetic polymers or triplets has already been reported by Dubnoff and Maitra [13], as well as by Wahba et al. [21]. In contrast, none of the F3 classes stimulate Phe-tRNA binding in the presence of poly U, thus showing no similarity with the FAB protein already described by Thach [22]. Whereas the same purified F3 subfractions appear to respond to Qβ, R17 mRNAs and to poly U, G the activity ratio with natural mRNA versus synthetic poly A, U, G or triplets varies considerably with the fraction tested. In particular, one protein fraction (F3b, tubes 12–15) is very efficient in stimulating fMet-tRNA binding in the presence of poly A, U, G or ApUpG, but displayed very little activity in the presence of natural mRNAs or poly U, G. This poly A, U, G or ApUpG-dependent activity of the tested F3 fractions could not be accounted for by a 70 S dissociating power, since the F3b preparation used had very little DF activity at the binding concentration.

It is presently difficult to assess whether the different classes of F3 "activities" thus observed correspond to different proteins, or to the same protein which could either complex with some other factor, or exist in various aggregated states leading to changes in its chromatographic behaviour and resulting in an inhibition of DF activity and of initiator tRNA binding stimulation with some messengers. This last possibility might perhaps account for the non-linearity when F3c activity is plotted against protein concentration. Another possibility would be that F3 classes are derived from the same protein after some proteolytic alteration which changes its properties: this is indeed the case with *E. coli* polynucleotide phosphorylase, for which endogenous proteolysis has been described [23].

The existence of factors specifically recognizing initiation triplets might be suggested by the fact that certain F3 fractions only respond to poly A, U, G or triplet ApUpG. The fact that certain F3 fractions recognize both natural mRNAs and triplets could be explained either by an aggregation between the first and the second category of translation proteins or by a dual recognition function of some F3. More work is obviously needed to clear up this point. Determination of the physico-chemical properties of the ApUpG recognition factor is indeed a pressing problem. One must also investigate whether the GpUpG recognition activity found in the factor specific for natural messenger is due to partial aggregation of the ApUpG recognition protein.

By and large it is tempting to assume that initiation proteins do exist, aside from F1 and F2, the function of which is : 1) to recognize the initiation triplets, and 2) to unfold the structure of natural mRNA so as to allow the attachment to the initial triplet or to the specific cistron. This would also be consistent with the reports that recognition of the initiation sequence changes under conditions where the conformation of mRNA is destroyed [24]. Existence of messenger- and even cistron-specific F3 factors, as suggested by Revel's observations [15], is supported by the different responses of F3b and F3c with T4 and R17 mRNA templates (table 1).

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